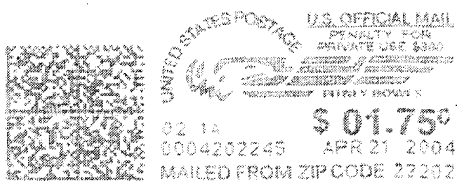
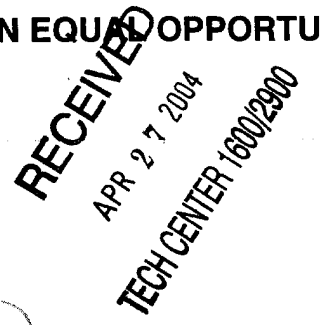
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/600,060	07/10/2000	Neil Andrew Williams	CTH-03	6761

7590 04/21/2004

Mary M Krinsky
79 Trumbull Street
New Haven, CT 06511-3708

EXAMINER	
HUYNH, PHUONG N	

ART UNIT	PAPER NUMBER
1644	

DATE MAILED: 04/21/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	09/600,060	WILLIAMS ET AL.	
	Examiner	Art Unit	
	Phuong Huynh	1644	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *Three* MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *02 February 2004*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *49,53-56,59-64,66,68-73,75-77 and 79-83* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *49,53-56,59-64,66,68-73,75-77 and 79-83* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date *8/27/03*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

1. Claims 49, 53-56, 59-64, 66, 68-69, 71-73, 75-77, and 79-83 are pending.

2. The following new ground of rejection is necessitated by the amendment filed 2/2/04.

3. The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

4. Claims 49, 53-56, 59-64, 66, 68-69, 71-73, 75-77, and 79-83 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling only for a method for treating a subject for asthma, allergic rhinitis, atoptic eczema, dermatitis, uticaria, hives comprising administering to the subject an effective amount of an agent wherein the agent is selected from the group consisting of Etx, Ctx, EtxB, and CtxB that bind to GM1 wherein the agent is administered with an allergen and is not coupled to said allergen, **does not** reasonably provide enablement for a method for treating a subject for any Type I allergy such as insect bite allergy, dietary allergy and drug allergies comprising administering to the subject a therapeutically effective amount of an agent such as Etx, Ctx, EtxB and CtxB alone that bind to GM1 or modifies any GM1 associated activity (claims 49, 53-55, 66, and 68) or with *any* antigen/allergen and not coupled to *any* antigen (claims 56, 61, 69, 71-73, 75-77, 79-83). The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in **scope** with these claims.

Factors to be considered in determining whether undue experimentation is required to practice the claimed invention are summarized *In re Wands* (858 F2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988)). The factors most relevant to this rejection are the scope of the claim, the amount of direction or guidance provided, the lack of sufficient working examples, the unpredictability in the art and the amount of experimentation required to enable one of skill in the art to practice the claimed invention. The specification disclosure is insufficient to enable one skilled in the art to practice the invention as broadly claimed without an undue amount of experimentation.

The specification discloses only a method of treating asthma, allergic rhinitis, atoptic eczema, dermatitis, uticaria, hives comprising administering to the subject an effective amount of an agent wherein the agent is selected from the group consisting of Etx, Ctx, EtxB, and CtxB that binds to GM1 wherein the agent is administered *with* an allergen and is not coupled to said allergen. The specification further discloses the use of Etx, Ctx, EtxB, CtxB for screening for agent that binds to GM1 and GM1 associated activity in vitro.

The specification does not teach how to treat a subject for *any* Type I allergy such as insect bite allergy, dietary allergy and drug allergies comprising administering to the subject a therapeutically effective amount of an agent that bind to GM1 or modifies any GM1 associated activity such as Etx, Ctx, EtxB and CtxB *alone*. There is insufficient guidance and in vivo working example demonstrating that administering Etx, Ctx, EtxB or CtxB *alone* without the allergen is effective for inducing tolerance to all undisclosed antigen/allergen, drug let alone for treating all type I allergy such as asthma, allergic cough, allergic rhinitis, conjunctivitis, atopic eczema, dermatitis, uticaris, hives, inset bit allergy, dietary allergy and drug allergies.

Even if the method of treating Type I allergy by coadministering to the subject an effective amount of an agent selected from the group consisting of Etx, Ctx, EtxB, or CtxB that binds to GM1 wherein the agent is administered with an antigen/allergen and is not coupled to said antigen, there is insufficient guidance and in vivo working examples demonstrating that the claimed method is effective for treating inset bit allergy, dietary allergy and drug allergies. Further, the term "antigen" without the amino acid sequence has no structure.

Kagan *et al* teach presently, the only available treatment of food allergies is dietary vigilance and administration of self-injectable epinephrine (abstract, in particular).

Wiedermann *et al* teach suppressive versus stimulatory effects of allergen/cholera toxoid (CtB) conjugates depending on the nature of the allergen in which murine model of type I allergy as well as the route of administration (See abstract, in particular). In the absence of guidance as to the structure of "antigen/allergen", the route of immunization and in vivo working examples, it is unpredictable which undisclosed antigen/allergen when coadminister to a patient is efficacious for inducing immune tolerance.

Herz *et al* teach allergens can differ in their immunogenicity as well as in their capacity to act as tolorogens (See abstract, page 274, nature of the antigen, in particular). Herz *et al* teach until now no mouse model has been available which resembles all of human bronchial asthma (page 272, column 2, Animal models of type I allergy and asthma, in particular). Each individual

mouse strain demonstrates a unique response pattern following immunization of allergens. The same allergen causes different phenotype dependent on genetical prerequisites (page 273, column 1, in particular). Further, the route of allergen administration has important impact on the quality of the immune response (See page 273, column 2, in particular). Herz et al teach that dependence of experimental model and the antigen used, the effects as well as the mechanisms of action can vary which might indicate the complexity of predicting clinical consequences of any therapeutic approach (see page 279, in particular).

Tamura et al (of record) teach that the physical association of LTB and antigen such as OVA is required to mediate immune suppression (See page 228, column 1, Figure 2, in particular).

Further, the term "modulating" could be inhibitory or stimulatory, which actions are mutually exclusive. There is insufficient guidance as to which GM1 associated activity is stimulatory and which GM1 associated activity is inhibitory upon administering the agent to the subject, in turn, effective for treating all type I allergy.

Reasonable correlation must exist between the scope of the claims and scope of enablement set forth. Even if the agent is limited to Etx, there is no showing in the specification as filed that said agent could treat *all* allergic disorders such as food allergy, drug allergy, insect bites, and contact dermatitis using a model that is specific for asthma. "It is not sufficient to define the recombinant molecule by its principal biological activity, e.g. having protein A activity, because an alleged conception having no more specificity than that is simply a wish to know the identity of any material with that biological property." Colbert v. Lofdahl, 21 USPQ2d, 1068, 1071 (BPAI 1992).

For these reasons, it would require undue experimentation of even one skilled in the art to practice the claimed invention. See page 1338, footnote 7 of Ex parte Aggarwal, 23 USPQ2d 1334 (PTO Bd. Pat App. & Inter. 1992).

In re wands, 858 F.2d at 737, 8 USPQ2d at 1404 (Fed. Cir. 1988), the decision of the court indicates that the more unpredictable the area is, the more specific enablement is necessary. In view of the quantity of experimentation necessary, the limited working examples, the unpredictability of the art, the lack of sufficient guidance in the specification and the breadth of the claims, it would take an undue amount of experimentation for one skilled in the art to practice the claimed invention.

Applicants' arguments filed 2/2/04 in conjunction with the declaration under 37 C.F.R. 1.132 filed 6/13/03 by Neil Andrew Williams have been fully considered but are not found persuasive.

Applicants' position is that (1) amended claims no refer to Type I allergy only. (2) claims 49, 56, 61 and 76 have been further amended to no longer refer to antibodies, and derivatives of antibodies. (3) The declaration by Neil Andrew Williams shows working example to treating asthma, a type I allergy. As long as the specification discloses at least one method for making and using the claimed invention that bears reasonable correlation to the entire scope of the claim, then the enablement requirement is satisfied.

However, the specification does not teach how to treat a subject for *all* Type I allergy such as insect bite allergy, dietary allergy and drug allergies comprising administering to the subject a therapeutically effective amount of an agent such as Etx, Ctx, EtxB and CtxB *alone* that bind to GM1 or modified which GM1 associated activity. There is insufficient guidance and in vivo working demonstrating that administering Etx, Ctx, EtxB or CtxB *alone* without the allergen is effective for inducing tolerance to all undisclosed antigen/allergen, drug, insect bites. Even if the method of treating Type I allergy by coadministering to the subject an effective amount of an agent selected from the group consisting of Etx, Ctx, EtxB, or CtxB that binds to GM1 wherein the agent is administered with an antigen/allergen and is not coupled to said antigen, there is insufficient guidance and in vivo working examples demonstrating that the claimed method is effective for treating inset bit allergy, dietary allergy and drug allergies. Further, the term "antigen" without the amino acid sequence has no structure. Kagan *et al* teach presently, the only available treatment of food allergies is dietary vigilance and administration of self-injectable epinephrine (abstract, in particular).

Wiedermann *et al* teach suppressive versus stimulatory effects of allergen/cholera toxid (CtB) conjugates depending on the nature of the allergen in which murine model of type I allergy as well as the route of administration (See abstract, in particular). The data provided in the declaration under 37 CFR 1.132 filed 6/13/03 by Neil Andrew Williams is limited to treating asthma using only EtxB.

Herz *et al* teach allergens can differ in their immunogenicity as well as in their capacity to act as tolorogens (See abstract, page 274, nature of the antigen, in particular). Herz *et al* teach until now no mouse model has been available which resembles all of human bronchial asthma (page 272, column 2, Animal models of type I allergy and asthma, in particular). Each individual

mouse strain demonstrates a unique response pattern following immunization of allergens. The same allergen causes different phenotype dependent on genetical prerequisites (page 273, column 1, in particular). Further, the route of allergen administration has important impact on the quality of the immune response (See page 273, column 2, in particular). Herz et al teach that dependence of experimental model and the antigen used, the effects as well as the mechanisms of action can vary which might indicate the complexity of predicting clinical consequences of any therapeutic approach (see page 279, in particular). Further, the term "modulating" could be inhibitory or stimulatory, which actions are mutually exclusive. There is insufficient guidance as to which GM1 associated activity is stimulatory and which GM1 associated activity is inhibitory upon administering the agent to the subject, in turn, effective for treating all type I allergy.

5. No claim is allowed.

6. Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, THIS ACTION IS MADE FINAL. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

7. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Phuong Huynh "NEON" whose telephone number is (571) 272-0846. The examiner can normally be reached Monday through Friday from 9:00 am to 5:30 p.m. A message may be left on the examiner's voice mail service. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Christina Chan can be reached on (571) 272-0841. The IFW official Fax number is (703) 872-9306.

8. Any information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Phuong N. Huynh, Ph.D.
Patent Examiner
Technology Center 1600
April 19, 2004

CHRISTINA CHAN
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

Notice of References Cited	Application/Control No. 09/600,060	Applicant(s)/Patent Under Reexamination WILLIAMS ET AL.	
	Examiner Phuong Huynh	Art Unit 1644	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

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	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Kagan et al, Environ Health Perspect 111(2):223-5, Feb 2003.
	V	Wiedermann et al, International Immunology 11(7): 1131-38, 1999
	W	Herz et al, Methods 32(3): 271-80, March 2004
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

1644

OIPE JC95 AUG 27 2003 PATENT & TRADEMARK OFFICE

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PATENT

00833-P0018A SPM/MWK

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	Neil Andrew Williams, *et al.*
Serial No. 09/600,060	Filing Date: July 10, 2000
Title of Application:	Agent for Treating Allergic or Hypersensitivity Condition
Confirmation No. 6761	Group Art Unit: 1644
Examiner	Phuong Huynh

Commissioner for Patents
Post Office Box 1450
Alexandria, VA 22313-1450

Information Disclosure Statement by Applicants

Dear Sir:

As a means of complying with the duty of disclosure set forth in 37 CFR §1.56, Applicants list the following references (copies of the listed patents and papers enclosed).

Foreign Patent Documents			
Exam. Initials	Document No.	Date	Country
PNH	WO 98/47529	Oct. 29, 1998	PCT

4/13/04

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August 25, 2003

Michael Krenicky

Food Allergy: An Overview

Rhoda Sheryl Kagan

Divisions of Allergy and Clinical Immunology and Rheumatology, Department of Pediatrics, Montreal Children's Hospital, McGill University Health Centre, Montreal, Quebec, Canada

Food allergy affects between 5% and 7.5% of children and between 1% and 2% of adults. The greater prevalence of food allergy in children reflects both the increased predisposition of children to develop food allergies and the development of immunologic tolerance to certain foods over time. Immunoglobulin (Ig) E-mediated food allergies can be classified as those that persist indefinitely and those that are predominantly transient. Although there is overlap between the two groups, certain foods are more likely than others to be tolerated in late childhood and adulthood. The diagnosis of food allergy rests with the detection of food-specific IgE in the context of a convincing history of type I hypersensitivity-mediated symptoms after ingestion of the suspected food or by eliciting IgE-mediated symptoms after controlled administration of the suspected food. Presently, the only available treatment of food allergies is dietary vigilance and administration of self-injectable epinephrine. ***Key words:*** **epidemiology, food allergy, IgE-mediated hypersensitivity.** *Environ Health Perspect* 111:223–225 (2003). [Online 21 January 2003]
doi:10.1289/ehp.5702 available via *http://dx.doi.org/*

As many as 30% of American adults self-report food allergy and alter their eating habits accordingly (Sloan and Powers 1986). Similarly, nearly one-third of parents perceive adverse food reactions to be responsible for a multitude of symptoms in their children and modify their children's diets in response (Bock 1987). Although accurate and recent epidemiologic data are scarce, current estimates of the prevalence of food allergy suggest that approximately 5% of young children and 1–2% of adults have reproducible symptoms resulting from food allergy (Bock 1987; Niestijl et al. 1994). Despite greater awareness and recognition of food allergy by both physicians and patients, many allergists believe that the actual prevalence has risen substantially over the past decade, similar to the rise in prevalence of other atopic conditions such as asthma and allergic rhinitis (Ninan and Russell 1992; Peat et al. 1994; Sears 1996).

An adverse reaction to food refers to any abnormal reaction after the ingestion of food or food additives. Adverse reactions to foods may result from enzyme deficiencies such as lactose intolerance, exaggerated pharmacologic responses to natural or added chemical agents such as vasoactive amines in wines, or immunologic responses. Immunologic responses to foods can be further defined mechanistically as immunoglobulin (Ig) E mediated and non-IgE mediated. The best-characterized adverse reactions to food are those that are Type I hypersensitivity reactions, i.e., IgE mediated. The spectrum of food allergy ranges from cutaneous symptoms, such as atopic dermatitis, appearing several hours after the ingestion of the responsible food to potentially life-threatening symptoms occurring immediately upon ingestion. Additionally, some individuals experience allergic symptoms only if the food is eaten before specific physical stimuli (for example, vigorous exercise) or if the individual has concomitant seasonal allergies, whereby certain foods elicit oral symptoms, such as pruritis and local swelling upon ingestion. This article is limited to classic type I hypersensitivity allergic reactions to foods.

Diagnosis

The diagnosis of food allergy rests with the detection of food-specific IgE and a history compatible with IgE-mediated symptoms occurring within an acceptable time frame (usually < 1 hr) after the isolated ingestion of the food in question. Symptoms that result from IgE-mediated mast cell degranulation products include flushing, urticaria, stridor or wheeze, and gastrointestinal symptoms such as abdominal pain or vomiting. Detection of food-specific IgE can be measured either with a skin prick test or, *in vitro*, with food-specific IgE. Skin prick tests are inexpensive, simple tests and can be performed in individuals of all ages. Skin prick tests have excellent sensitivity and negative predictive value, but variable specificity and positive predictive values; therefore skin tests, particularly when performed with fresh foods, can reliably exclude food allergies when negative, but cannot confirm food allergy when positive (Bock et al. 1978; May 1976; Sampson 1988). When measured with the ImmunoCAP method (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden), a fluoroenzymatic immunoassay, quantitative measures of food-specific IgE can be determined, and these measurements can be useful in the diagnosis of food allergy. The positive and negative predictive values of a limited number of food-specific IgE determinations have been published that define cut-off values that reliably predict the likelihood of food allergy when compared with double-blind, placebo-controlled, food challenges (DBPCFC) (Sampson 2001). The DBPCFC is the "gold standard" of food allergy diagnosis and requires the administration of incremental quantities of the suspected food to the patient in a controlled, blinded, and supervised setting, where observation and documentation of objective allergic signs are noted. The procedure and safety of DBPCFC have been described elsewhere (Bock et al. 1988). Thus, the diagnosis of food allergy can be made when the history is convincing of an IgE-mediated reaction and confirmatory skin tests or specific IgE assays are positive. However, when the history elicited is not clearly typical of an IgE-mediated reaction, either by description of symptoms or in timing, or when the meal that produced the reaction contained several allergic possibilities, positive skin tests or specific IgE measurements must be confirmed with DBPCFC before an accurate diagnosis of food allergy is made. Clearly, when the history and lab tests are convincing of food allergy, the DBPCFC is not indicated.

Natural History of Food Allergy

Most food allergies have their onset in infancy or early childhood, depending on when the food is introduced into the diet. Theoretically, any food containing a protein could elicit an allergic reaction; however, eight common foods are responsible for > 90% of food allergies (Hefle et al. 1996). The natural history of food allergy varies with the individual food, but many of the foods that elicit allergic reactions in young children can be eventually reintroduced into the diet. Thus, most food allergies can be classified as "likely to resolve" or "likely to persist." Food allergies that usually resolve include milk, soy, egg, and wheat. These allergies typically present in infancy and usually resolve by school age. Food allergies that usually persist include peanut, tree nuts, fish, and shellfish. These, too, usually present in early childhood, shortly after the introduction of these foods into the usual diet. Although most individuals with

Address correspondence to R. Kagan, The Montreal Children's Hospital, 2300 Tupper Street, Room C-510, Montreal, Quebec H3H 1P3 Canada. Telephone: (514) 412-4470. Fax: (514) 412-4390. E-mail: rhoda.kagan@muhc.mcgill.ca

This article is part of the mini-monograph "Animal Models to Detect Allergenicity to Foods and Genetically Modified Products."

Received 9 April 2002; accepted 2 October 2002.

allergies to foods in this latter group tend to persist with these allergies indefinitely, some children will develop tolerance to these foods and will be able to reintroduce them safely into their diet.

Milk allergy almost always presents in the first year of life, soon after the introduction of cow's milk or cow's milk–based infant formula, and usually resolves by school age. Most infants with cow's milk allergy develop gastrointestinal symptoms, approximately 50–70% have cutaneous features, and about 20–30% will have respiratory symptoms (Host 1994). Milk allergy affects up to 2.5% of infants, with approximately 1% of all children developing IgE-mediated milk allergy and approximately 1.5% of children developing non-IgE-mediated milk allergy (Host and Halken 1990). In a prospective study by Host and Halken (1990), 39 infants in a birth cohort of 1,749 unselected newborns were diagnosed with cow's milk protein allergy/intolerance based on elimination diets and milk rechallenges. Of 39 infants diagnosed with milk allergy or milk intolerance, 21 infants had positive skin or serum IgE tests to milk and 18 had non-IgE-mediated cow's milk intolerance. Of the total group, 56% were able to tolerate milk by 1 year of age, 77% by 2 years, and 87% by 3 years. Of children with IgE-mediated cow's milk allergy, 14% had persistent milk allergy at the age of 5 and 10 years. All of the children with non-IgE-mediated cow's milk allergy and most children with IgE-mediated cow's milk allergy were able to reintroduce milk products by age 5; however, 3 of 21 of the IgE-mediated group remained allergic well into mid-childhood. Thus, in an unselected population, milk allergy usually resolves by school age, but among highly atopic children, milk allergy is more likely to persist (Bishop et al. 1990; Tikkanen et al. 2000).

Risk factors for persistence of milk allergy include early dermatitis presentation of milk allergy; development of other atopic conditions, including other food allergies, asthma, and allergic rhinitis; and persistence of elevated levels of milk-specific IgE (Sicherer and Sampson 1999; Zeiger et al. 1999). In general, as tolerance to IgE-mediated milk allergy is achieved, the size of the wheal and flare on the skin prick tests decreases; however, it may continue to remain positive beyond acquisition of clinical tolerance. Serum-specific IgE, measured by the ImmunoCAP system, appears to be a more sensitive measure for the prediction of food allergy resolution, especially in patients with atopic dermatitis (Sampson 2001).

Soy is considered a major food allergen and is a food introduced to infants in the form of infant formulas and cereals. Soy-based infant formulas are recommended for families following vegetarian dietary restrictions, for children with congenital or acquired lactose intolerance, and for infants with diagnosed IgE-mediated cow's milk allergy. Of children with IgE-mediated cow's milk allergy, fewer than 15% will develop a concomitant allergy to soy, but most infants will tolerate soy protein without difficulty (Zeiger et al. 1999). The prevalence of soy allergy/soy intolerance varies with the frequency with which soy is introduced into regional diets, but it appears to affect 1–6% of infants (Giampietro et al. 1997; Magnolfi et al. 1996). Symptoms associated with soy allergy include typical IgE-mediated features as well as non-IgE-mediated—gastrointestinal symptoms such as hematochezia and malabsorption. Both skin prick tests and food-specific IgE are used to detect the presence of IgE; however, both modalities have poor specificity and positive predictive values (Giampietro et al. 1997; Sampson 2001). The natural history of IgE-mediated soy allergy is similar to that of other "predominantly transient" allergies, and most children can tolerate soy products by school age.

The prevalence of egg allergy is estimated at 1.6–2.6% of the general pediatric population but is significantly higher among individuals with atopic dermatitis and other collateral atopic conditions (Danneus et al. 1977; Eggesbo et al. 2001). Most children developing allergic symptoms to ingestion of egg develop symptoms within 30 min. More than 85% of egg-allergic children develop cutaneous symptoms, 60% have gastrointestinal symptoms, and up to 40% will have associated respiratory symptoms (Eggesbo et al. 2001; Ford and Taylor 1982). In Ford and Taylor's (1982) description of the natural history of egg allergy, 44% of egg-allergic children were able to reintroduce egg products into their diet by school age, but the remaining 56% persisted with egg allergy. Children with persistent egg allergy had significantly more target organs affected at the time of the initial allergic reaction, were more likely to acquire additional atopic conditions, and continued to have positive prick skin tests to egg. Egg-specific IgE, measured by the ImmunoCAP method, is useful in predicting the likelihood of positive challenges, and cutoff values have been proposed for this effect (Sampson 2001).

Peanuts, nuts, fish, and seafood allergies generally persist indefinitely. Other foods, particularly seeds (e.g., sesame, poppy, mustard), might also be added to this list. Uncommonly, reports of tolerance developing to these foods have been published, but until very recently, the persistence of these food allergies has been expected.

Peanut allergy deserves particular attention because it almost always presents early in life, is often severe, generally persists indefinitely, and is the most common cause of fatal food-related anaphylaxis (Bock and Atkins 1989; Sampson et al. 1992). Additionally, because of peanut's relative ubiquity, accidental exposures occur frequently, despite vigilant attempts to avoid peanut-containing foods (Bock and Atkins 1989; Vander Leek et al. 2000). The prevalence of peanut allergy is approximately 0.6%, and there is some evidence that this has increased (Grundy et al. 2002; Sampson 1996; Sicherer et al. 1999; Tariq et al. 1996). Tariq et al. (1996) described the prevalence of peanut allergy and peanut sensitization in a birth cohort of children born between 1989 and 1990. The prevalence of sensitization to peanut was 1.1% and the prevalence of confirmed peanut allergy was 0.5%. In 2001, a similar birth cohort, born between 1994 and 1996, was prospectively followed for evidence of peanut sensitization and allergy. The sensitization prevalence had increased to 3.2%, and the peanut allergy prevalence rose to 1.5% (Grundy et al. 2002).

Approximately 80% of peanut-allergic children develop allergic symptoms at the time of their first known exposure to peanut, > 90% develop symptoms within 30 min of ingestion, 90% have cutaneous features, 40% have respiratory symptoms, and 50% develop allergic manifestations to contact alone (Hourihane et al. 1997; Sicherer et al. 1998). After diagnosis and despite avoidance measures, most peanut-allergic children have accidental exposures to peanut resulting in allergic symptoms, and > 40% of subsequent allergic reactions may be more severe than the initial reaction (Bock and Atkins 1989; Vander Leek et al. 2000). However, in a subset of peanut-allergic children, up to 20% will become tolerant to peanut and will be able to reintroduce peanut into their diets (Hourihane et al. 1998; Skolnick et al. 2001; Spergel et al. 2000). Factors that appear to predict resolution of peanut allergy include mild cutaneous allergic features at onset, fewer associated atopic features, loss or diminution of skin prick test reactions to peanut, and low levels of peanut-specific IgE.

Studies on the natural history of allergies to other foods considered to be "lifelong" are scant. Although it does appear that most adults with shellfish allergy remain allergic to crustaceans, reports of individuals becoming tolerant to shrimp have been published (Daul et al. 1990). Likewise, it is possible that tolerance to other "persistent" food allergies may develop in some individuals, but publications provide little guidance in determining which patients may resume eating these foods. In the interim, patients with allergies to nuts, seeds, fish, and crustaceans are advised to avoid ingesting these foods indefinitely, unless oral challenge tests demonstrate acquisition of tolerance.

Treatment

The treatment of food allergy is limited to encouraging strict dietary vigilance and the ability to self-treat an allergic reaction if it were to occur. Maintaining dietary vigilance is difficult and stressful but can reduce the likelihood of experiencing an accidental ingestion of the allergic food (Bock and Atkins 1989; Ewan and Clark 2001; Primeau et al. 2000; Vander Leek et al. 2000). Currently, the only available therapy for food allergy is avoidance and self-treatment with auto-injectable epinephrine. Early administration of epinephrine is life-saving, and proper technique should be taught to all food-allergic individuals (Sampson et al. 1992). Promising immunotherapy interventions are being developed to diminish the severity of life-threatening food allergies but are not yet commercially available.

Conclusion

Food allergies affect between 5% and 8% of the pediatric population and between 1% and 2% of the adult population and appear to be rising. Many food allergies are "outgrown" during childhood, but some individuals never develop tolerance to some of the more commonly seen "transient" food allergens and remain symptomatic indefinitely. Factors associated with the persistence of "transient" food allergies include early onset, greater severity of allergy expression, collateral atopic conditions, persistence of skin prick test reactions, and elevated food-specific IgE. Conversely, a minority of individuals with allergy to foods traditionally believed to persist indefinitely have demonstrated loss of the allergy and clinical tolerance. Further research into the risk factors and associated clinical and laboratory tests that may predict persistence or resolution of food allergy may help to characterize this dichotomy.

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Suppressive versus stimulatory effects of allergen/cholera toxoid (CTB) conjugates depending on the nature of the allergen in a murine model of type I allergy

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Abstract

Recent reports have demonstrated that feeding small amounts of antigen conjugated to the B subunit of cholera toxin (CTB) suppress immune responses in experimental models of certain T_h1-based autoimmune diseases. We have established a model of aerosol sensitization leading to T_h2-mediated allergic immune responses in BALB/c mice. In the present study two different antigens, the dietary antigen ovalbumin (OVA) and the inhalant allergen Bet v 1 (the major birch pollen allergen), chemically coupled to recombinant CTB were tested for their potential to influence T_h2-like immune responses. Intranasal administration of OVA–CTB prior to sensitization with OVA led to a significant decrease of antigen-specific IgE antibody levels, but a marked increase of OVA-specific IgG2a antibodies as compared to non-pretreated, sensitized animals. Antigen-specific lympho-proliferative responses *in vitro* were reduced by 65% in the pretreated group; IL-5 and IL-4, but not IFN-γ, production were markedly decreased in responder cells of lungs and spleens of nasally pretreated mice. In contrast, mucosal administration of rBet v 1–CTB conjugates prior to sensitization led to an up-regulation of allergen-specific IgE, IgG1 and IgG2a, increased *in vitro* lympho-proliferative responses as well as augmented production of IL-5, IL-4, IL-10 and IFN-γ. Intranasal administration prior to sensitization of unconjugated allergens showed also contrasting effects: OVA could not significantly influence antigen-specific antibody or cytokine production, whereas intranasal pretreatment with unconjugated Bet v 1 suppressed allergen-specific immune responses *in vivo* and *in vitro*. These results demonstrated that the two antigens—in conjugated as in unconjugated form—had different effects on the T_h2 immune responses. We therefore conclude that the tolerogenic or immunogenic properties of CTB—and probably also other antigen-delivery systems—strongly depend on the nature of the coupled antigen–allergen.

Introduction

It is well established that mucosal administration of soluble antigens induces systemic immunological unresponsiveness, a phenomenon known as mucosal tolerance (1,2). However, it has been recognized that the effectiveness of mucosal tolerance often requires repeated and large amounts of antigen, and sometimes the suppressed immune responses are of short duration. On the other hand, molecules with known mucosa-binding properties are known to induce local and systemic immune responses when administered by the mucosal routes (3). Among these, cholera toxin (CT) is

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one of the most potent mucosal immunogens, which, when administered simultaneously with an antigen by the mucosal route, enhances immune responses to the co-administered antigen (4). In contrast to cholera holotoxin, mucosal administration of only the non-toxic, mucosa-binding B subunit of CT (CTB), physically coupled to an antigen, has been recently recognized to enhance peripheral tolerance induction (5). Sun *et al.* demonstrated that a single feeding of antigen conjugated to CTB led to suppression of T cell responses *in vivo* and *in vitro* at doses 15- to 500-fold lower than at those of corresponding regimes using unconjugated antigens (5). In line with this finding further studies have demonstrated the effectiveness of such antigen–CTB formulations in preventing or treating certain T_h1-based diseases, such as experimental autoimmune encephalomyelitis (6) or spontaneous autoimmune diabetes (7). Recently, a similar transmucosal carrier-delivery system, the enterotoxin B of *Escherichia coli* conjugated to antigen, has been successfully used for suppression not only of antigen-specific T cell responses but also of antigen-specific IgE production (8). So far, this is the only study providing evidence that such a mucosal antigen-delivery system might be a promising strategy to suppress immune responses, based on excessive T_h2 cell activity.

Type I allergy is very common in areas of the temperate climate zone and the prevalence of the disease has constantly increased within the last years (9). This genetically determined immunodisorder, manifested by symptoms like allergic rhinitis, conjuctivitis or allergic asthma, is based on the excessive production of IgE antibodies against allergenic molecules (10). The pathway of IgE regulation is well described for mice and humans, and essentially explained by the reciprocal activity of IL-4 and IL-5, mediating T_h2 responses, and IFN-γ, which antagonizes these effects (11–13).

We have established a murine model of aerosol sensitization leading to allergen-specific T_h2-mediated immune responses i.e. high IgE/IgG1 versus low IgG2a antibody levels and positive type I skin tests *in vivo*, an immunological state comparable to that of human type I allergy. Using this model system we have recently reported that simultaneous inhalation of allergen and cholera holotoxin can modulate the allergic immune response in naive as well as in sensitized animals (14).

In the present study we investigated, whether mucosal delivery of antigen–CTB conjugates could also be successfully used to suppress type I allergic immune responses. Two different antigens were used for this purpose: ovalbumin (OVA), a frequently experimentally used dietary antigen, and recombinant Bet v 1, the major allergen of birch pollen (BP) and a clinically important inhalant allergen (15,16).

Methods

Animals

Female, 7-week-old, BALB/c mice were obtained from Charles River (Sulzfeld, Germany). All experiments were approved by the Animal Experimentation Ethics Committee of the University of Vienna and the Ministry of Science and Research.

Antigens

Recombinant Bet v 1 (rBet v 1) was purchased from Biomay (Linz, Austria).

BP was obtained from Allergon (Engelholm, Sweden) and used for the preparation of a BP extract as previously described (14).

Recombinant CTB was produced in a mutant strain of *Vibrio cholerae* deleted from the CTA subunit gene and transfected with a plasmid encoding CTB. CTB was purified by sequential precipitation and gel filtration chromatography (17,18). OVA (Sigma, St Louis, MO) and rBet v 1 were covalently bound to CTB at a molar ratio of 1:1 using *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) as bifunctional coupling reagent, according to the protocol described by Sun *et al.* (5). The resulting OVA–CTB and rBet v 1–CTB conjugates were purified by gel filtration through a column of Sephacryl S-300. The conjugates were shown to retain GM1 binding capacity and serological activity of both OVA and rBet v 1 by means of solid-phase ELISA (19).

Nasal administration of CTB-coupled proteins

Mice (n = 6/group) were anesthetized and 40 µg/30 µl NaCl of the OVA–CTB (20 µg OVA conjugated to 20 µg CTB) or rBet v 1–CTB (20 µg rBet v 1 coupled to 20 µg CTB) were intranasally applied. For control purposes corresponding amounts of unconjugated OVA or rBet v 1 or NaCl were intranasally applied to mice (n = 6/group). This procedure was performed at three occasions at 7 day intervals prior to sensitization (days –21, –14 and –7; Fig. 1 experimental design).

Sensitization

Pretreated and sham-pretreated mice were once injected i.p. with 50 µg OVA or 1 µg rBet v 1 adsorbed to $Al(OH)_3$ (day 0). Fourteen days later aerosol exposure to a 0.1% OVA or BP solution was daily performed during a period of 2 times over 5 days (days 14–18 and 21–25) as previously described (14). The OVA and the BP solution were aerosolized using a nebulizer (nebulizer 646; Devilbiss, Sommerset, PA). The mice were exposed to 4 ml solution for 20 min/day (Fig. 1, experimental design).

Sampling

Blood samples were taken before sensitization and 6 days after the last aerosol exposure. Serum was prepared and stored at –20° C until analyzed.

Bronchoalveolar lavage (BAL) was collected by lavaging the lungs with 1 ml PBS inserted through a small incision in the trachea of the sacrificed mice (14).

Immunoblots

Proteins were separated by SDS–PAGE and transferred to nitrocellulose by electroblotting according to standard protocols (20,21). Sera from OVA and Bet v 1/BP immunized mice were incubated in 1/5 (IgE) and 1/100 (IgG1 and IgG2a) dilutions with nitrocellulose stripes coated with OVA or BP extract. Immunoblots were performed with rat anti-mouse IgE (1/250), IgG1 (1/500) or IgG2a (1/250; PharMingen, San Diego, CA) followed by radiolabeled sheep anti-rat Ig (1/1000; Amersham, Little Chalfont, UK).

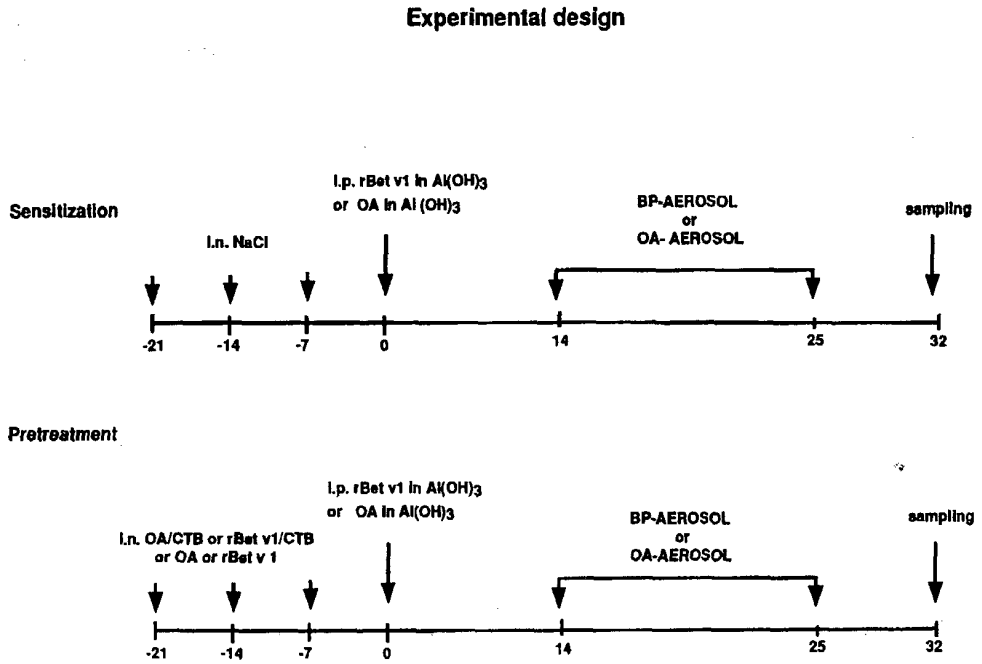


Fig. 1. Experimental design. Two groups of control mice (n = 6/group) were either i.p. immunized with OVA/Al(OH)$_3$ or with rBet v 1/Al(OH)$_3$ and subsequently aerosolized with an 0.1% OVA or BP solution. Before sensitization 0.9% NaCl (sham-pretreatment) was intranasally applied. Four groups of mice (n = 6/group) were 3×intranasally treated with OVA–CTB, rBet v 1–CTB, unconjugated OVA or unconjugated rBet v 1. Thereafter the mice were immunized with OVA or rBet v 1/BP as described for the control mice.

Determination of antigen-specific antibody levels in serum and bronchial lavage

Microtiter plates (Nunc, Roskilde, Denmark) were coated with OVA (10 µg/ml carbonate buffer) and BP extract (50 µg/ml). Serum samples were diluted 1/1000 for IgG1, 1/500 for IgG2a and 1/10 for IgE; BAL was used undiluted. Rat anti-mouse IgG1, IgG2a, IgE and IgA antibodies (1 µg/ml; PharMingen) and thereafter peroxidase-conjugated mouse anti-rat IgG antibodies (1/1000; Jackson ImmunoResearch, PA) were used. Color development was performed as previously described (14).

Lymphocyte proliferation assay

At sacrifice, 7 days after the last aerosol exposure (day 32), spleens and lungs with associated lymph nodes were removed under sterile conditions. The organs were homogenized, erythrocytes were lysed, and the cells washed and resuspended in complete medium (RPMI, 10% FCS, 0.1 mg/ml gentamycin, 2 mM glutamine and 50 µM 2-mercaptoethanol). Suspensions of spleen cells as well as lung cells with associated lymph nodes were plated into 96-well round-bottom plates at a concentration of 2×10^5/200 µl/well and stimulated with and without concanavalin A (Con A; 0.5 µg/well), OVA (40 µg/well) or BP (5 µg/well) for 4 days. Thereafter the cultures were pulsed with 0.5 µCi/well [^{3}H]thymidine (Amersham) for 16 h and the proliferative responses measured by scintillation counting.

Measurement of cytokine production in spleen and lung cell cultures

For determination of IL-5, IL-4, IL-10 and IFN-γ production spleen and lung cells were cultured with and without Con A (2.5 µg/well), OVA (100 µg/well) and BP (25 µg/well) in 48-well plates (Costar, Cambridge, MA, USA) at a concentration of 5×10^6/0.5 ml/well. After 24 and 48 h supernatants were taken and stored at –20° C until analyzed.

The levels of IL-5, IL-4, IL-10 and IFN-γ were measured with mouse ELISA kits (Endogene, Cambridge, MA). The sensitivity of the IL-5 and IL-4 assay was <5 pg/ml and of the IL-10 and IFN-γ ELISA <15 pg/ml.

Statistics

For statistical analysis the Mann–Whithney U-test was used.

Results

Sensitization

Mice, immunized i.p. with OVA or Bet v 1 adsorbed to Al(OH)$_3$ and subsequently exposed to an OVA or BP solution via aerosol, displayed high allergen-specific IgG1/IgE versus low IgG2a antibody levels. Figure 2(A) demonstrates the binding of serum IgE, IgG1 and IgG2a antibodies from OVA-sensitized mice to the 40 kDa molecule. Mouse sera from Bet v 1/BP immunized mice only bound to the 17 kDa Bet v 1 molecule

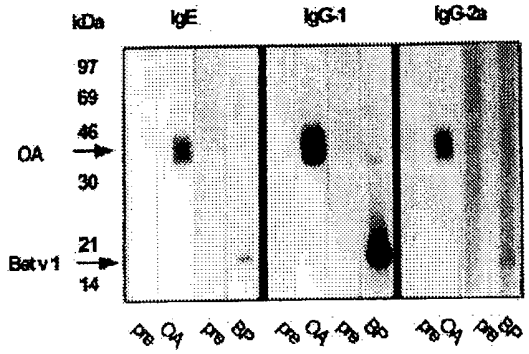


Fig. 2. Immunoblot, performed with pooled sera of mice immunized according to the standard immunization protocol, i.e. 1×i.p. injection of $Al(OH)_3$ adsorbed OVA or Bet v 1, followed by aerosol exposure to 0.1% OVA or BP solution. (pre) Nitrocellulose stripes incubated with pooled preimmune sera. (OVA) Binding of serum IgE, IgG1 and IgG2a from OVA sensitized mice to the 40 kDa OVA molecule. (BP) Binding of serum IgE, IgG1 and IgG2a of Bet v 1/BP immunized mice to Bet v 1, the 17 kDa major BP allergen. Additional IgG1 binding to the 34 kDa Bet v 1 dimer.

(Fig. 2B). Therefore, all tests performed with BP extract reflect the immune reactivity to Bet v 1 exclusively.

Mucosal administration of OVA–CTB conjugate leads to suppression whereas application of rBet v 1–CTB conjugates augments T_h2-like immune responses in vivo

Antigen-specific antibody levels in serum and BAL were measured in mice treated 3 times intranasally with OVA–CTB or unconjugated OVA or 3 times intranasally pretreated with rBet v 1–CTB conjugates or unconjugated rBet v 1 and compared with those of OVA- or Bet v 1-sensitized control mice.

As shown in Fig. 3(A) mucosal pretreatment with OVA–CTB conjugates led to a significant reduction of OVA-specific IgE antibody levels but simultaneously to a drastic increase of anti-OVA IgG2a antibody levels. IgG1 anti-OVA antibody levels (Fig. 3 A) as well as IgA anti-OVA levels in BAL did not differ between the sensitized (IgA-OD: 0.279 ± 0.23) and the nasally pretreated mice (IgA-OD: 0.219 ± 219). Intranasal administration of equivalent doses of unconjugated OVA could not markedly decrease antigen-specific IgE nor significantly increase IgG2a antibody levels (Fig. 3B).

In total contrast to the pretreatment with OVA–CTB conjugates, intranasal administration of rBet v 1–CTB conjugate prior to sensitization led to enhanced antibody responses of all isotypes examined. In particular, IgE, IgG1 and IgG2a antibodies against BP/Bet v 1 were significantly higher in the conjugate pretreated group as compared to the sensitized animals ($P < 0.01$, Fig. 3A). A similar increase in allergen-specific IgA antibody levels in BAL was noted in the conjugate pretreated animals (OD: 1.0 ± 0.7) compared to the controls (OD: 0.087 ± 0.05). To investigate, if the enhanced immune responses were due to CTB, unconjugated rBet v 1 was intranasally applied before sensitization. This pretreatment led to a strong reduction of the allergen-specific antibody responses of all isotypes as shown in Fig. 3(B).

Pretreatment with OVA–CTB conjugates leads to suppression, whereas rBet v 1–CTB conjugates cause enhancement of antigen-specific lympho-proliferative responses in vitro

Antigen-specific lympho-proliferative responses were measured in lung and spleen cell cultures of mice pretreated with antigen–CTB conjugate or unconjugated antigen and compared with cultures derived from sensitized animals. Lympho-proliferative responses in lung cells of OVA–CTB pretreated mice were reduced by 65% [stimulation index (SI): 2 ± 1.0] compared to the sensitized mice (SI: 5.7 ± 4.7, $P < 0.05$). An even stronger reduction (81%) of the lympho-proliferative response was noted in the group of mice pretreated with unconjugated OVA (SI: 2.1 ± 2.3) compared to the sensitized mice (SI: 11.3 ± 4.4, $P < 0.02$). However, in spleen cell cultures no differences in the antigen-specific proliferative responses were observed between the groups (Fig. 4).

In contrast, intranasal administration of rBet v 1–CTB prior to sensitization with BP led to a 273% increase of lympho-proliferative responses of lung cell cultures (SI: 5.6 ± 5.2) and a 163% enhanced response of spleen cells (SI: 5.1 ± SD 3.5) as compared to the sensitized animals (SI: 1.5 ± SD: 0.7 respectively SI: 1.9 ± SD: 0.3) (Fig. 4).

However, intranasal administration of unconjugated Bet v 1 resulted in a 37–44% reduction of allergen-specific lympho-proliferative responses in lung (SI: 1.5 ± 0.2) and spleen cells (SI: 2.0 ± 0.3) as compared to the sensitized animals (SI: 2.4 ± 0.9 respectively SI: 3.6 ± 1.7) (Fig. 4).

Pretreatment with OVA–CTB decreases production of IL-5 and IL-4, whereas rBet v 1–CTB leads to increased cytokine production in vitro

In vitro production of IL-5, IL-4, IL-10 and IFN-γ was measured in spleen and lung cell cultures of sensitized and pretreated animals. Intranasal administration of OVA–CTB conjugates prior to sensitization led to reduced IL-5 and IL-4 production in cultures of pooled spleen and lung cells compared to the sensitized controls (Table 1). IFN-γ and IL-10 levels of did not markedly differ between the groups. No significant differences in IL-5 and IFN-γ production were observed in cell cultures of mice pretreated with unconjugated OVA compared to OVA sensitized mice (Table 1).

In contrast, pretreatment with rBet v 1–CTB led to a drastic increase of IL-5, IL-4, IFN-γ and IL-10 production in spleen and lung cell cultures compared to the cytokine levels measured in cultures of the sensitized control animals (Table 1). This augmented immune response *in vitro* was consistent with the enhanced allergen-specific antibody responses *in vivo* (Fig. 3).

The opposite effects were obtained from mice intranasally treated with unconjugated Bet v 1 prior to sensitization. In line with the suppressed antibody production (Fig. 4) also the *in vitro* production of IL-5, IL-4, IL-10 and IFN-γ was markedly reduced compared to that of the sensitized animals (Table 1).

Discussion

In the present study we demonstrated that the use of antigen–CTB conjugates had opposite effects on T_h2-like immune

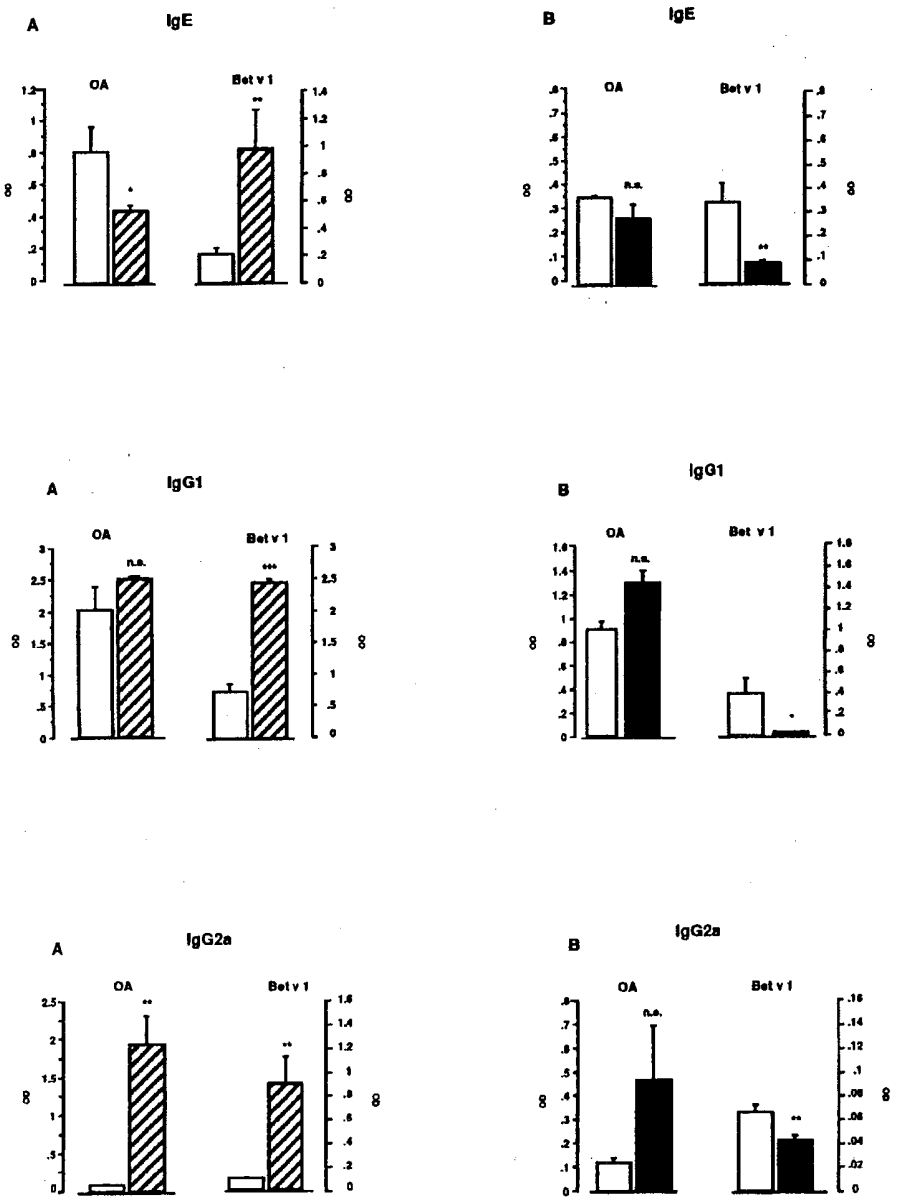


Fig. 3. Serum IgE, IgG1 and IgG2a antibody responses against OVA or Bet v 1 measured by ELISA. (A) White bars represent the mean OD of six control mice immunized with OVA or Bet v 1/BP according to the standard protocol. Hatched bars represent the mean OD of six mice, intranasally treated with OVA–CTB or rBet v 1–CTB prior to sensitization. (B) White bars represent the mean OD of six control mice immunized with OVA or Bet v 1/BP. Black bars represent the mean OD of six mice, intranasally treated with unconjugated OVA or unconjugated rBet v 1 prior to sensitization. Error bars show SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, Mann–Whitney *U*-test.

responses depending on the antigen coupled to CTB. Intranasal administration of OVA–CTB conjugates prior to sensitization suppressed, whereas administration of rBet v 1–CTB conjugates stimulated immune responses *in vivo* and *in vitro*.

Previous reports have demonstrated that mucosal administration of soluble antigens conjugated to CTB is more effective than that of unconjugated antigens to induce peripheral tolerance in naive as well as sensitized animals. These studies, mainly performed in models of T_h1- mediated diseases, consistently reported about the ability of antigen–CTB conjugates to reduce delayed-type hypersensitivity reactions or T cell responses *in vitro* (5,6,7,22).

Less data exist about the suppressive effects on T_h2-like immune responses. A recent study showed that CTB conjugated to the *Leishmania* antigen LACK or OVA selectively reduced T_h1 responses (IFN-γ production), whereas T_h2 responses (IL-4/IL-5 production) remained unaffected by this treatment. It was therefore suggested that the selective toler-

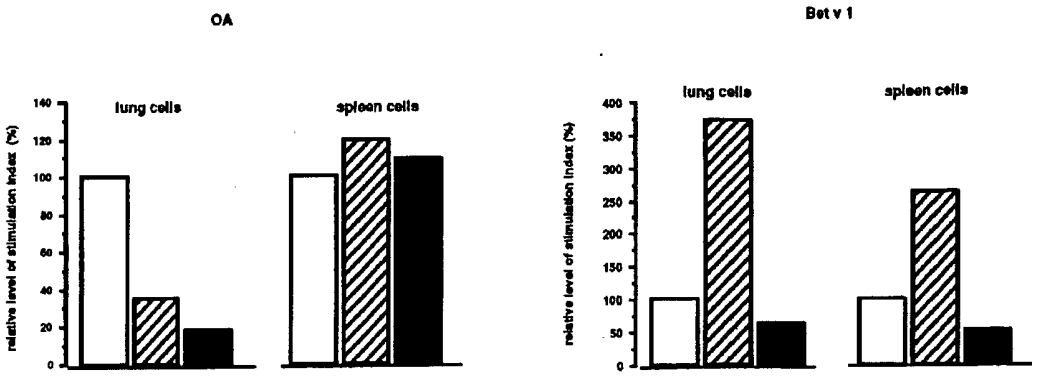


Fig. 4. Relative levels of stimulation indices of responder cells from lung and spleen cell cultures, stimulated with OVA or BP antigen. The white bars represent the proliferative response of sensitized mice, hatched bars of antigen–CTB pretreated mice and black bars of mice pretreated with unconjugated antigen prior to sensitization. The stimulation index of the sensitized mice was set to 100% and the stimulation indices of the pretreated groups were expressed as relative values.

Table 1. *In vitro* cytokine production in spleen and lung cell cultures

Spleen cells					
Pretreatment	Sensitization	IL-5	IL-4	IL10	IFN-γ
NaCl	OA	39.6	4.8	8.9	n.d.
OA/CTB	OA	18	5	19.7	n.d.
NaCl	Bet v 1/BP	2.3±0.9	10.3±7.2	72.3±25.2	75.9±48.0
rBet v 1/CTB	Bet v 1/BP	141.2±115.5**	28.2±22.1 n.s.	319.8±193**	1517.9±10633**
NaCl	OA	68.8±10.7	n.d.	n.d.	309.0±370.2
OA	OA	56.9±67.8 n.s.	n.d.	n.d.	234.8±414.3 n.s.
NaCl	Bet v 1/BP	81.7±170	44±97.2	108±65.16	1041.6±931.3
rBet v 1	Bet v 1/BP	2.4±4.1*	1.7±2.4 n.s.	68.5±49.5 n.s.	17.1±14.5**
Lung cells					
Pretreatment	Sensitization	IL-5	IL-4	IL10	IFN-γ
NaCl	OA	86.5	8.1	18.9	90
OA/CTB	OA	11.4	4.7	20	120
NaCl	Bet v 1/BP	22.2±28	5.7±13	5.3±9	7.7±11.8
rBet v 1/CTB	Bet v 1/BP	135±197*	3.3±3.9	56.8±39**	103.5±110
NaCl	OA	272±53.3	n.d.	n.d.	7.7±11.8
OA	OA	163±197 n.s.	n.d.	n.d.	62.5±114.5 n.s.

Concentrations of cytokines in supernatants of spleen- and lung cell cultures (n = 6/group) of mice intransally pretreated or sham- (NaCl) pretreated before sensitization. IL-5 and IL-4 production (pg/ml) was measured after 24 hours, IL-10 and IFN-γ (pg/ml) after 48 hours stimulation with antigen. Values represent the mean of 6 individual cell cultures of each group (± standard deviation). In the experiment of OA/CTB pretreated and OA sensitized mice spleen and lung cells were pooled within each group. n.d. = not done, n.s. = non significant, * $P< 0.05$, ** $P< 0.01$.

ance of T_h1 cells was due to a common effect of CTB rather than an antigen-specific effect of the proteins conjugated to CTB. The authors concluded that such therapeutical strategies might therefore preferentially be used for treatment of T_h1-based immunopathological situations (22). However, another study recently reported that a similar antigen delivery system—the bacterial product LTB coupled to OVA—could suppress both T_h1 and T_h2 responses, as demonstrated in a decreased delayed-type hypersensitivity reaction as well as suppressed OVA-specific IgE antibody responses (8).

In the present study we used our murine model of aerosol sensitization to investigate the potential of two different antigens, the dietary antigen OVA and the inhalant allergen Bet v 1, coupled to CTB for their potential to affect T_h2-like immune responses. Our present data demonstrated that intranasal pretreatment with OVA–CTB conjugates led to a significant

reduction of OVA-specific IgE antibody levels. We also noted a significant increase in antigen-specific IgG2a antibody production, indicating the capacity to drive the immune response towards a T_h1-like response (Fig. 3A). The reciprocal activities of IL-5/IL-4—crucial for induction of T_h2 responses—and IFN-γ known to control the production of IgG2a in the murine system—is well documented (12,23). In accordance with the antibody responses the *in vitro* synthesis of IL-5/IL-4, but not of IFN-γ, was markedly decreased in lung and spleen cell cultures of OVA–CTB pretreated mice (Table 1). This immunomodulating effect was due to the CTB component of the conjugate, since intranasal application of comparable amounts of unconjugated OVA could not reduce IgE or enhance IgG2a antibody levels (Fig. 3B), nor significantly modulate the cytokine production profile (Table 1). Our data indicates that T_h2 responses can be influenced by intranasal administration of OVA–CTB. This is in contrast to the study of McSorely *et al.*, reporting that pretreatment with OVA–CTB conjugates via the same route failed to affect T_h2 immune responses (22).

Notably, using rBet v 1–CTB conjugates under identical experimental conditions resulted in a general stimulation of allergen-specific antibody production of all isotypes in serum (Fig. 3A) and a concomitant increase of IgA in BAL. T cell proliferative responses and the cytokine production *in vitro* (IL-5, IL-4, IFN-γ and IL-10) were also significantly enhanced in cell cultures (Fig. 4 and Table 1). In contrast to Bet v 1 coupled to CTB, intranasal application of unconjugated Bet v 1 led to a significant reduction of antibody responses of all isotypes (Fig. 3B), T cell proliferation (Fig. 3B) as well as cytokine production *in vitro* (Table 1). These observations demonstrated that, in the case of Bet v 1, CTB acted as an efficient adjuvant.

It has been demonstrated that the method of conjugation can influence the effect of such a transmucosal delivery system in terms of suppression or stimulation of the immune responses. These different effects have been explained by possible alterations of the antigen structure due to different methods of conjugation (8,24). Since we used the same method of conjugation for both antigens, our study indicates that the nature of the antigen *per se* influenced the quality of the immune response. It has been suggested that certain allergens have intrinsic abilities—in addition to acting as an immunogen—to intervene with regulatory processes involved in the development of allergy. Such intrinsic activities can be due to enzymatic properties (25–27) and/or certain molecular/structural features of the allergen. In the latter respect, Bet v 1 shows conformational epitopes, which can be destroyed by producing two parts of the molecule (28), whereas OVA is known to possess only continuous epitopes (29). These structural differences of the two molecules might have been responsible for the different outcome of the treatment with conjugated as well as unconjugated Bet v 1 compared to OVA.

In conclusion, considering CTB as a potential antigen-delivery system in T_h2-based immunopathological conditions—such as type I allergy—the above described formulation might only be successfully applicable with certain allergens. Consequently, the effects of novel adjuvants/antigen-delivery systems cannot be generalized based on experiments performed with model antigens, but need to be individually evaluated.

Acknowledgements

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Abbreviations

BAL	bronchoalveolar lavage
BP	birch pollen
Bet v 1	major birch pollen allergen
Con A	concanavalin A
CT	cholera toxin
CTB	cholera toxin B subunit
OVA	ovalbumin
r	recombinant
SI	stimulation index

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Erratum

Suppressive versus stimulatory effects of allergen/cholera toxoid (CTB) conjugates depending on the nature of the allergen in a murine model of type I allergy

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The Publishers apologize for the fact that this paper was first published without the authors' corrections. The corrected version follows.

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Animal models of type I allergy using recombinant allergens

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Abstract

Various animal models including guinea pigs, monkeys, dogs, rats, and mice have been established in an attempt to provide insights into the complex immunological and pathophysiological mechanisms of human type I allergic diseases. The detailed knowledge of the murine genome, the various components of the murine immune system, and the generation of engineered mice has made the murine system the most attractive among all animal models. The availability of multitude technologies and reagents to characterize and manipulate immunological pathways and mediators adds to the outstanding opportunities to assess the pathology of allergic diseases and to develop novel therapeutic strategies in mice. Numerous sensitization protocols with food and aero-allergens are used to establish an allergic/asthma-like phenotype in mice. Requirements for an appropriate murine model include a close resemblance to the pathology of the disease in humans, the objective measurement of the physiologic parameters, as well as reliability and reproducibility of the experimental data. With respect to reproducible experimental conditions, it has been recognized that extract preparations from natural allergen sources can vary in their allergen-content and -composition. This might influence the degree of sensitization or the outcome of treatment strategies in dependence of the applied extract preparation. The use of recombinant allergens in experimental in vivo and in vitro systems can overcome these problems. Another aspect, that has become obvious from the experimental studies, is that allergens can differ in their immunogenicity as well as in their capacity to act as tolerogens. Therefore, it seems important that the efficacy of the different allergen-molecules to act as therapeutic agents is individually examined. In this review, examples of animal models are described, in which recombinant allergens have been used for sensitization and/or treatment of allergic responses and how they have been used to enhance our understanding of the pathology of allergic diseases.
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1. Background

1.1. Type I allergy and asthma

Allergic diseases such as bronchial asthma are involving complex interactions between exogenous factors such as allergen exposure, infections and other environmental stimuli, and genetically determined factors. Bronchial asthma is a chronic inflammatory disease defined by chronic airway inflammation, reversible broncho-obstruction, and non-specific airway hyperresponsiveness (AHR). Sensitization to normally harmless environmental antigens (pollen, house-dust mite, etc.) is thought to be the prerequisite for initiating the inflammatory cascade in bronchial asthma. Repeated and continuous allergen-aerosol exposure causes inflammation of the airway mucosa and submucosa, which is orchestrated by Th-2 type T-cells. These cells produce Th-2 cytokines (IL-4, IL-5, IL-9, IL-13, and IL-15), which regulate both IgE production and airway inflammation. Particularly, airway inflammation is thought to be the main underlying pathological aspect of bronchial asthma, characterized by the influx of eosinophils and activated CD4+ T-cells. Tissue damage due to eosinophil degranulation products—and as a consequence airway mucosa remodelling—may contribute to severity of the disease and development of AHR. An ideal treatment of already established bronchial asthma should cure the disease. At present, as for most multifactorial and chronic conditions, this has not been achieved. Another important approach represents primary and secondary prevention. Normally, environmental allergens are recognized as

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harmless antigens. The appropriate immune response pattern can be described as development of T-cell tolerance. Inability to either develop or maintain tolerance will then result in the development of Th-2 mediated inflammation in a susceptible individual. The immune system offers a wide range of mechanisms to prevent or suppress such dysregulations including induction of counter-active Th-1 cells, CD-8 suppressors, regulatory T-cells, and production of anti-inflammatory/suppressive cytokines, including IL-10 and TGF-β.

1.2. Current treatment against type I allergy in humans

Specific immunotherapy (SIT) is performed by injection of increasing amounts of allergen extracts to induce hyporesponsiveness to the respective allergens. Even though SIT is an effective treatment in many cases of allergic rhinitis and in allergy to insect venom, certain drawbacks, such as the long duration of the treatment or the risk of anaphylactic side reactions, have to be taken into account. Moreover, the use of allergen extracts encounters the possibility of de novo sensitization against components present in natural allergen preparations. Therefore, the concept of a component resolved, patient-tailored therapy using the disease-eliciting allergen in recombinant form, as well as a change to less invasive routes have been suggested for enhanced treatment efficacy [1,2].

The mechanisms operating in SIT are still not completely understood. It seems that several immunological pathways are involved in the clinical improvement achieved by the common SIT schedules: (I) rise in allergen-specific IgG antibodies, in particular IgG4, which is supposed to block the allergen and IgE-facilitated allergen presentation to T-cells [3–5]; (II) generation of IgE-modulating CD8+ T-cells [6]; (III) reduction in the number of mast cells, eosinophils, and release of mediators [7,8]; and (IV) modulation of allergen-specific T cells: a shift from Th-2 to Th-1 cytokine pattern with a decrease of IL-4 and IL-5 production accompanied by an increase of IFN-γ (immune deviation) [9–11]; (V) moreover, the induction of an anergic state in peripheral T-cells (immunologic tolerance), which is mediated by IL-10 and characterized by suppressed proliferative and cytokine responses against major allergens, represents an important aspect in successful SIT [12].

1.3. Mucosal tolerance

Mucosal tolerance describes a state of an antigen-specific non-responsiveness of the immune system induced by mucosal application of soluble antigen. Based on the physiological role to inhibit hypersensitivity reactions towards harmless antigens—such as environmental inhalant allergens, food or the commensal bacterial flora—mucosal tolerance induction has been suggested as a treatment strategy against several inflammatory diseases. In that respect experimental studies have demonstrated the efficacy of mucosal antigen administration in preventing the onset or severity of certain Th-1-based autoimmune diseases [13–15] and in suppressing Th-2 based allergic immune responses [16–18].

The mechanisms of tolerance induction seem to be very complex and depend on many factors, such as the nature of the antigen, antigen-dose, and frequency of application. High-dose antigen administration preferentially leads to T-cell anergy or deletion [19–21]. Application of low antigen-doses favours the induction of regulatory T-cells, of which several subtypes with distinct cytokine secretion pattern of TGF-β and IL-10 have been described [22–24]. Additional data suggest also a role of CD8+ Tr cells in tolerance induction [25]. Antigen-presenting cells, in particular dendritic cells (DCs) seem to play an important role in determining the outcome of an immune response: in dependence of the maturation state [26,27] and/or the phenotype of DCs [28] antigen presentation leads to suppressive immune responses or induction of inflammatory signals. Recently, distinct DC populations from different mucosal compartments were described, showing that gastrointestinal tract-DCs preferentially lead to induction of TGF-β producing Th3 cells, whereas DCs from the respiratory tract favour the activation of IL-10 producing Tr-1 cells [29,30].

2. Animal models of type I allergy and asthma

An ideal mouse model for human type I allergic diseases such as bronchial asthma should resemble the major features of the human disease including high levels of allergen-specific immunoglobulin production, immediate hypersensitivity with early and late phase responses, airway inflammation characterized by the influx of T-cells, eosinophils and some neutrophils, development of airway hyperresponsiveness, and chronic airway remodelling with deterioration in lung function. Until now no mouse model has been available, which resembles all of these features of human bronchial asthma. Especially the development of chronic airway inflammation and airway remodelling is not yet reached. In mice, exposure to allergens induces allergen-specific immunoglobulin production associated with the development of immediate cutaneous hypersensitivity reactions after allergen challenge. In addition, after repeated airway allergen challenges these mice develop acute but not persistent airway inflammation, including eosinophil accumulation and both, airway hyperresponsiveness to unspecific stimuli such as methacholine and acute broncho-obstruction during allergen-aerosol exposure [31,32]. However, the level and quality of such a Th-2

biased immune response depend largely on the genetic background of the mice, the route, dose and frequency of exposure to the allergen, and the type of adjuvant being used.

2.1. Genetic background (Table 1)

As in humans, the establishment of a full-type asthmatic syndrome in mice seems to be highly dependent on the genetic background of the used mouse strain. Based on the level of allergen-specific IgE/IgG1 production and the degree of airway inflammation following repeated airway allergen challenges, high- and low-responder mouse strains were identified. It becomes apparent that each individual mouse strain demonstrates a unique response pattern following immunization to allergens. In many studies, either BALB/c or C57BL/6 mice were used. BALB/c mice are known as IgE-high responders to many allergens (e.g., ovalbumin, OA), whereas C57BL/6 and SJL mice are characterized as intermediate and low-IgE responder animals (Table 1). All strains develop cutaneous immediate-type hypersensitivity reactions due to mast cell degranulation triggered by cross-linking of membrane-bound allergen-specific IgE and IgG1 antibodies after allergen challenge. However, the inflammatory component of the lung and the severity of altered lung function differ between the strains [33]. In OVA-sensitized BALB/c mice, a marked increase in lymphocytes, eosinophils, and neutrophils in bronchoalveolar lavage (BAL fluids) is paralleled by elevated production of IL-4, IL-5, and TNF-α in the lung. In contrast, in OVA-sensitized C57BL/6 mice, the inflammatory immune response in the lung is much weaker. Moreover, BALB/c mice demonstrated greater degree of airway hyperreactivity as C57BL/6 mice, indicating that the same allergen causes different phenotypes dependent on genetical prerequisites. Although the recruitment of eosinophils to the airways is a central feature of all mouse models of asthma, and although this cell population has been linked to the development of the asthma phenotype, the contribution of eosinophils to the development of altered airway function remains controversial. Indeed, several studies with wild-type and knock-out mice implicate IL-4 as the key cytokine and suggest that increased airway resistance (AR) can occur independently of IL-5 and eosinophils [34–37], whereas other studies provide equally convincing evidence that IL-5 is the critical cytokine [38–41]. Furthermore, it has been shown that passive sensitization of BALB/c mice with allergen-specific IgE and IgG1 antibodies alone is sufficient to induce a state of increased AR and airway inflammation following airway allergen challenges [42]. Recently, it was demonstrated that early phase bronchoconstriction requires allergen-specific IgG1, but is independent of allergen-specific IgE and mast cell activity [43].

2.2. Protocols of allergic sensitization and airway inflammation

Sensitization to inhalant and/or food allergens determines the first phase in a dynamic set of events leading to allergic bronchial asthma. In mice, a wide variety of soluble proteins are used to establish an allergen-specific IgE/IgG1 immune response, which is associated with the development of cutaneous immediate-type hypersensitivity responses and airway inflammation. However, several factors directly influence the quality and quantity of the immune response to the allergen and differences in the protocols can have drastic effects on the development of the asthma-like phenotype in mice, and as a consequence appropriate interpretation of the results becomes difficult. As a model-allergen ovalbumin, a dietary allergen in humans is widely used in animal experiments, in addition to a variety of several clinical important inhalant allergens—such as those from birch and grass pollen or house-dust mite allergen, and pet allergen—used for allergic sensitization [44]. The route of allergen administration has an important impact on the quality of the immune response. The highest immune response is achieved by a combination of systemic sensitization, to induce peripheral priming of the immune response, and repeated airway allergen challenges to induce airway inflammation. This is achieved by systemic (subcutaneous, intraperitoneal) injection(s) of antigen/allergen adsorbed to the adjuvants aluminium hydroxide $Al(OH)_3$, long known to induce Th-2

Table 1
Development of immediate type I hypersensitivity responses to allergens, airway inflammation, and altered lung function in different mouse strains

Mouse strain	MHC class-II	OVA	Birch pollen	House-dust mite	Ragweed
BALB/c	H-2^d	High	High	Low	Low
C57BL/6	H-2^b	Intermediate	Intermediate	High	High
CBA	H-2^k	Intermediate	n.d.	High	High
SJL	H-2^s	Low	n.d.	Low	n.d.

Mice of different strains were sensitized by intraperitoneal injection of the allergen on days 1, 14, and 21. On days 26 and 27, mice received two consecutive airway allergen challenges by aerosolization of the allergen as previously described. Development of an asthma-like phenotype was assessed by measurement of allergen-specific serum IgE/IgG1 antibody titers, assessment of airway inflammation, determination of airway responsiveness to unspecific stimuli, and analysis of skin test responses. Animals were considered as "high responder" mice if all read-out systems gave positive results. Low responders were mice that did not mount a specific IgE response and had normal airway responses.

responses, followed by multiple airway allergen challenges. The allergen could be delivered either by aerosolization of the allergen or instillation by intratracheal or intranasal application. Such protocols result in a maximal and reproducible induction of an inflammatory immune response in the airways paralleled by development of airway hyperresponsiveness.

That systemic priming prior to mucosal antigen application is essential for induction of allergic immune responses has become obvious from studies by Holt et al., showing that antigen inhalation alone led to a preferential suppression of antigen-specific IgE antibodies [16,45]. The phenomenon of peripheral tolerance induction via mucosal surfaces has been recognized early in animal models after oral antigen application and ever since then it has been referred to as oral/mucosal tolerance [46].

2.3. The nature of the antigen

In the majority of animal models ovalbumin (OVA) has been used as a model antigen to study pathophysiological events and the mechanisms behind experimental and clinical immunomodulations. In particular, the availability of OVA-transgenic mice has proved to be of great advantage in studies on the inductive and effector sites of immunostimulation and suppression [47].

However, with respect to experimental models of allergic asthma, sensitization with an inhalant rather than a dietary allergen may be closer to the situation in humans. Moreover, it is well recognized that different antigens can vary in their immunogenicity as well as their capacity to act as tolerogens. As an example, we have previously noticed that OVA, applied under identical experimental conditions as the birch pollen allergen Bet v 1, was less immunogenic (in conjunction with a mucosal adjuvant) and tolerogenic than the inhalant allergen [48]. Therefore, it seems necessary that the efficacy of each allergen molecule/construct to act as potential therapeutic agent is individually examined in a suitable model of type I allergy.

2.4. Role of adjuvants

Adjuvants are frequently used in conjunction with different vaccines, including SIT. Apart from the immunoenhancing effects, certain adjuvants can also influence the quality of an immune response. Parenteral antigen administration in conjunction with $Al(OH)_3$ is known to promote a Th-2 response and is therefore a commonly used adjuvant for induction of Th-2 immune responses. In mice, these responses are characterized by the appearance of allergen-specific IgE and IgG1 antibodies in blood, associated with the production of Th-2 type cytokines of primed lymphocytes. Certain bacterial compounds [49,50], bacterial DNA [51,52], or CpG-oligonucleotides [53,54] rather induce Th-1 responses, which belong to a new generation of immunomodulators, examined for potential use for treatment of type I allergy. Counterbalancing Th-2 inflammatory reactions can also be achieved through infection or vaccination, which stimulates the Th-1 development. Mouse experiments suggested a protective effect of *Bacillus* Calmette–Guérin (BCG) vaccination [55–57]. Although findings do not necessarily implicate a role of BCG vaccination in preventing allergy in humans, limited studies indicated that *M. vaccae* treatment in adults with asthma and rhinitis or in children with atopic dermatitis demonstrated clinical benefits as measured by reduction in use of rescue medication, severity of disease or inhibition of the allergen-induced late-phase response [58,59].

In the case of mucosally applied antigens, the use of certain mucosal adjuvants is required to induce any proper immune response. Among these, cholera toxin (CT), the enterotoxin of *Vibrio cholerae*, is one of the most potent experimentally used adjuvants [60]. We have previously shown that aerosol-treatment of allergen together with the mucosal adjuvant cholera toxin induced a Th-1 response in naïve mice and modulated an allergic immune response in sensitized mice. We proposed that also certain mucosal adjuvants could be useful tools for treatment strategies against allergies [61].

3. Animal models with recombinant allergens (Table 2)

3.1. Pollen allergy

Birch pollen allergy. We have previously established a murine model of allergic sensitization to birch pollen. BALB/c mice were identified as high IgE-responders to the major birch pollen allergen Bet v 1. The standard sensitization scheme is based on an intraperitoneal injection of recombinant (r) Bet v 1 adsorbed to $Al(OH)_3$, followed by an aerosol challenge with the whole birch pollen extract. This sensitization procedure leads to high allergen-specific IgE and IgG1 levels, associated with positive type I skin tests in vivo, as well as high IL-4 and IL-5 versus low IFN-γ production in vitro in response to birch pollen. Moreover, these mice develop airway inflammation, characterized by eosinophilia and IL-5 levels in lungs and bronchial alveolar lavage (BAL), as well as airway hyperresponsiveness [62,63]. Using this model immunomodulation was achieved either by injection of the immunodominant peptide [64] or by mucosal application of rBet v 1 prior to or after sensitization. Intranasal or oral application of rBet v 1 led to suppression of allergen-specific antibody levels of all isotypes, reduction of IL-4, IL-5, and IFN-γ, as well as inhibition of airway inflammation and airway hyperresponsiveness in naïve and sensitized mice [65,66]. Low dose tolerance induction prior to sensitization lasted up

to a year and at least 6 months in mice tolerized after sensitization. This indicated that Bet v 1 acts as a strong tolerogen. Further experiments were carried out to investigate the underlying mechanisms of tolerance induction. It was found that the state of tolerance was associated with increased levels of TGF-β. Furthermore, tolerance could be transferred by CD4+ T-cells, and to a lesser extent also by CD8+ T-cells, indicating active suppression as a central operating mechanism [67] (see Table 2).

Grass pollen allergy. Unlike in birch pollen allergy, several allergens have been characterized as major allergens in grass pollen allergic patients. To analyse the allergenic potency of these allergens, BALB/c mice were immunized with recombinant Phl p 1, Phl p 2, Phl p 5 or Phl p 6. It was demonstrated that only those allergens that bind high levels of IgE in humans induced high IgE and IgG1 levels in mice, indicating that the immunogenicity of the allergens is similar in humans and mice. Moreover, repeated immunizations with single recombinant allergens led to induction of protective IgG1 antibodies, since these IgG1 antibodies strongly inhibited human IgE binding to the allergen and suppressed allergen-induced histamine release from human basophils [68].

Using recombinant grass pollen allergens for intranasal tolerance induction revealed that the tolerogenic property of an allergen was not closely related to its immunogenicity: in comparison to Bet v 1, tolerance induction with Phl p 5 only led to incomplete immunosuppression, whereas sensitization with Phl p 5 led to higher humoral and cellular immune responses as well as stronger airway inflammation than in Bet v 1 sensitized mice [69].

Other models of pollen-associated allergies have been established aiming at evaluating the immune responses to recombinant and natural Ole 1 in comparison to the sensitization pattern of olive pollen allergic patients [70], or to study the efficacy of peptide-based oral immunotherapy against Japanese cedar pollinosis [71].

3.2. House-dust mite allergy

A model of allergic asthma to house dust mite was established by systemic priming and subsequent intranasal challenge with minute amounts (1 μg) of the group 1 allergen of *Dermatophagoides pteronyssinus*, Der p 1 in C57BL/6J mice. This sensitization protocol led—in the absence of allergen-specific IgE—to extensive pulmonary eosinophilic inflammation, which shared many features of inflammation found in human asthma, such as eosinophilic influx into the large airways, mucus plugging, and formation of Charcot–Leyden crystals [72]. These results differed from our studies in the model of birch pollen allergy in BALB/c mice, where airway inflammation was only achieved by inhalation of high doses (mg amounts) of allergen and in the presence of IgE. These differences might be related to either distinct intrinsic properties of various allergens or to genetic differences in the mouse strains used to sensitize against house-dust mite and birch pollen.

It was shown further that intranasal administration of high doses of the immunodominant T- cell epitope of Der p 1 induced long-lasting tolerance. Tolerance to the immunodominant epitope inhibited T-cell responses to all other epitopes of Der p 1 in mice challenged with the intact protein. This phenomenon was termed "'linked suppression," mediated by CD4+ cells. The mechanism of linked suppression was not associated with suppressive or anti-inflammatory cytokines, such as TGF-β or IL-10, but with the Notch signalling pathway, that generally regulates cell differentiation through cell–contact dependent mechanisms. In this case, cell–cell contact via an interaction of Delta-1 expressed on inhibitory T-cells and its ligand Notch on naïve cells may result in transmission of negative signals preventing clonal expansion [73–75].

Recently, it was demonstrated that the route/type of sensitization can influence the effects of peptide induced tolerance. Only after systemic immunization with Der p 1, but not after respiratory immunization—subsequent to intranasal peptide tolerance induction—C56BL/6J mice exhibited reduced allergen-specific IgE; immunosuppression at the cytokine level (Th-1 and Th-2 responses) was however induced by either type of sensitization [76].

A murine model of type I allergy to the group 2 allergen of *Dermatophagoides farinae*, Der f 2, was established aiming at inducing tolerance by oral application of recombinant Der f 2 [77]. Very high oral doses (20 mg) of the recombinant allergen were necessary to suppress allergen specific immune responses as well as immediate airway constriction and neutrophil influx into the airways [77]. Intranasal treatment with recombinant Der f 2 or a mutant of Der f 2 was associated with a downregulation of CD23 expression on B-cells; however, no direct changes in allergen-specific IgE/IgG1 or Th2 cytokines, such as IL-4 or IL- 5, were observed [78].

3.3. Latex allergy

Mice have been shown to mount a dose and time dependent IgE response to latex proteins following topical, respiratory, and subcutaneous exposure [79]. Bronchoconstriction was only elicited after topical, intranasal or intratracheal, but not after systemic immunization with natural allergens [80]. These data indicated that different clinical manifestations of the allergic responses depend on the route of sensitization [81,82]. In another study using two major recombinant allergens of latex, rHev b 1, and rHev b 3, high levels of

allergen-specific IgE/IgG1 antibodies were induced by systemic immunization. Murine T cells recognized the same T-cell epitopes as human T-cell clones from latex allergic patients [83,84]. Intranasal tolerance induction prior to sensitization with the recombinant latex allergens led to suppression of allergen-specific IgG1/IgE levels and cytokine production (IL-4, IL-5, and IFN-γ) in vitro. In contrast to tolerance induction with the major birch pollen allergen Bet v 1, intranasal treatment with recombinant Hev b 1 or Hev b 3 was less effective in sensitized BALB/c mice, indicating that allergens have varying tolerogenic properties and require individual doses for successful treatment [84].

3.4. Cat allergy

Mice sensitized with Fel d 1, the major cat allergen, exhibited T-cell, B-cell, and mast-cell responses when challenged with the protein. Subcutaneous injections of peptides containing the immunodominant T-cell epitopes of the allergen induced T-cell tolerance in pre-sensitized mice. The fact that these tolerized mice produced a decreased amount of histamine in vivo, indicated that histamine release was not solely dependent on the reduction of allergen-specific IgE. These data showed that mast cell activity in mice with established type I allergy can be regulated through peptide-induced T-cell tolerance [85,86].

3.5. Bee venom/Wasp venom allergy

In CBA/J mice, sensitized with phospholipase A2 (PLA_2)—a major bee venom allergen—daily treatment for 6 days by i.p. injections with overlapping peptides, spanning the entire PLA_2 molecule, resulted in significant reduction of allergen-specific IgE and an increase in IgG2a antibodies. The marked T-cell unresponsiveness was characterized by a shift from a Th2 to a Th1 profile. Upon rechallenge with the native PLA_2, the treated mice were fully protected from anaphylaxis [87]. Also after intranasal application of the overlapping peptides of PLA2 an immunological hyporesponsiveness, associated with an enhanced IgG2a response, was induced. As underlying mechanism clonal anergy was discussed, affecting more profoundly the Th-2 responses [88]. Successful immunotherapy with T-cell epitope-peptides inducing T-cell anergy was recently shown in bee venom allergic patients [89].

Concerning wasp venom allergy we have sensitized BALB/c mice with *Vespula vulgaris* venom extract without the use of adjuvant, thereby mimicking the natural route of sensitization. Mucosal pretreatment with recombinant Ves v 5, one of the major allergens of wasp venom, led to immunosuppression not only against Ves v 5 but also against the whole wasp venom extract. Unlike in the studies with bee venom PLA_2, tolerance induction was not associated with a shift from Th-2 to Th-1 responses, but a general immunosuppression due to regulatory mechanisms [90].

4. New approaches for prophylaxis and therapy of type I allergy

4.1. Systemic immunization—induction of protective IgG

Allergen-derivatives with reduced allergenicity have been promoted to prevent anaphylactic side reactions during SIT [91]. In that respect, it was shown that immunization of mice with two recombinant fragments of Bet v 1, which failed to bind IgE due to a loss of B cell epitopes, induced the production of IgG antibodies that cross-reacted with the complete Bet v 1. The antibodies inhibited the IgE binding of allergic patients to the wild type allergen [92,93]. Similar results of induction of protective IgG antibodies were achieved after immunization of mice with peptides derived from B-cell epitopes of Phl p 1 [94], or with recombinant hybrid allergens of the major grass pollen allergens Phl p 1, Phl p 2, Phl p 5, and Phl p 6 [95].

Induction of non-anaphylactic blocking IgG antibodies has also been achieved using Bet v 1 mimotopes for immunization [96]. Conjugation of these mimotopes to monovalent fusion proteins not only generated IgG that could inhibit human IgE binding, but also presented to be safe candidates for therapy as these conjugates could not cross-link cell-bound IgE in sensitized mice [97].

4.2. Induction of mucosal tolerance

Hypoallergenic molecules have also been shown to be promising candidates for mucosal tolerance induction. Prophylactic treatment with a hypoallergenic fragment of Bet v 1 led to a comparably strong suppression of T and B cell responses and inhibition of airway inflammation as with the whole recombinant Bet v 1 molecule [67]. Recent data suggest that such fragments can also be successfully used for mucosal treatment of already sensitized mice [98]. The mechanism of tolerance induction seems to depend on the structure of the molecule, as fragment treated mice—in contrast to Bet v 1 treated mice—did not exhibit elevated TGF-β levels nor was tolerance transferable with CD4+ or CD8+ T cells [67].

Based on the fact that many allergic patients are sensitized to several unrelated allergens, tolerance induction with a panel of simultaneously applied allergens is a desirable goal. We have established a model of polysensitization with recombinant Bet v 1, Phl p 1, and Phl p 5. Intranasal tolerance with a mixture of the immunodominant peptides of the three allergens led to a marked decrease of the IgE/IgG2a ratio and a decrease in Th2 cytokines. Similarly as observed after treatment

Table 2
Examples for mouse models of type I allergy using recombinant allergens

Antigen	Mouse strain	Sensitization	Treatment	Effects		Reference
OVA	BALB/c	i.p. OVA/alum	Oral OVA prior sens.	IgE	–	Van Halteren et al. [114]
				IgG1	=	
				IFN-γ	+	
OVA	BALB/c	i.p. OVA/Al(OH)$_3$, OVA aerosol challenge	n.d.	IgG1/IgE		Renz et al. [115]
				IL-4/IL-5		Herz et al. [33]
				Airwayinflamm.		
				AHR		
Bet v 1	BALB/c	i.p. Bet v 1/Al(OH)$_3$; aerosol with birch pollen extract	i.n., oral rBet v 1 prior and after sens.	IgG1, IgE, IgG2a	–	Wiedermann et al. [48]
				Skin tests	–	
				IL-4, IL-5, IFN-γ	–	
				TGF-β	+	Winkler et al. [66]
				Airwayinflamm.	–	
				AHR-	–	
Bet v 1	BALB/c	i.p. Bet v 1/AlOH)$_3$; aerosol with birch pollen extract	i.n. Bet v 1- fragment prior sens.	IgG1, IgE, IgG2a	–	Wiedermann et al. [63]
				Skin tests	–	
				IL-4, IL-5, IFN-γ	–	
				TGF-β	–	
				Airwayinflamm.	–	
				AHR	+	
Bet v 1	BALB/c	i.p. Bet v 1/Al(OH)$_3$; aerosol with birch pollen extract	i.n., s.c., i.p. CpG-ODN + Bet v1 prior and after sens.	IgG1, IgE	=	Jahn-Schmid et al. [104]
				IgG2a	+	
				IFN-γ	+	
				IL-5, eosinophils	–	
Bet v 1	BALB/c	i.p. Bet v 1/Al(OH)$_3$	i.d. plasmid DNA (pCMV-Bet v1)+/– CpG-ODN	IgG2a/IgG1 ratio	+	Hartl et al. [101]
				IFN-γ	+	
Bet v 1	BALB/c	i.p. Bet v 1/Al(OH)$_3$	i.n. *L. plantarum*, *L. lactis* + Bet v 1 prior and after sens.	IgG2a/IgG1 ratio	+	Repa et al. [112]
				IFN-γ	+	
				IL-5/IFN-γ ratio	–	
Ole e 1/ Olive pollen	BALB/c	i.p. Ole e 1/Al(OH)$_3$	n.d.	IgG1, IgE IL-4		Batanero et al. [70]
Cry j 2/ Japanese cedar pollen	BALB/c	i.n. Cry j 2 + CT	Oral Cry j 2-peptide prior and after sens.	IgG1, IgE	–	Hirahara et al. [71]
				IL-2	–	
				IL-4	–	
				IFN-γ	–	
Der p 1	C57BL/6	s.c. Der p 1/Al(OH)$_3$; i.n. Der p 1	n.d.	Eosinophils, mucus plugging, Charcot-Leyden crystals in lungs		Clark et al. [72]
Der p 1	C57BL/6	s.c. Der p 1/FCA	i.n. peptides prior and after sens.	DTH	–	Hoyne et al. [73–75]
				IgE	=	
				IL-2, IFN-γ	–	
				IL-3, IL-4	–	
Der p 1	C57BL/6J	i.p. Der p 1/Al(OH)$_3$	i.n. peptide	IgE	–	Jarnicki et al. [76]
				IL-4, IL-5	–	
				IFN-γ	–	
		i.n. Der p 1 + LTB	i.n. peptide	IgE	=	Jarnicki et al. [116]
				IL-4, IL-5	–	
				IFN-γ	–	
Der f 2	A/J	i.p. Der f 2/FCA	Oral Der f 2 after sens.	IgG1, IgE	–	Yasue et al. [77]
		i.p. Der f 2/Al(OH)$_3$	i.n. peptides after sens.	Airwayinflamm.	–	
				CD23	–	Yasue et al. [78]
Latex allergen	BALB/c	topical, i.n., s.c. latex allergens	n.d.	IgE		Woolhiser et al. [81]
				Eosinophils		Meade et al. [82]
				AHR		Thakker et al. [80]

Table 2 (*continued*)

Antigen	Mouse strain	Sensitization	Treatment	Effects		Reference
Hev b 1, Hev b 3	BALB/c	i.p. Hev b 1, Hev b 3 /Al(OH)$_3$	i.n. Hev b1, Hev b 3	IgG1, IgE	–	Hufnagl et al. [69]
				IL-4, IL-5	–	
				IFN-γ	–	
Fel d 1	BALB/c	s.c. Fel d 1/Al(OH)$_3$	s.c. Fel d 1 peptides prior and after sens.	IL-4, IFN-γ	–	Briner et al. [85]
				Histamine- release	–	Treter et al. [87]
Bee venom	CBA/J	i.p. PLA$_2$/Al(OH)$_3$	i.p. PLA$_2$ peptides or i.n. PLA$_2$ peptides prior and after sens.	IgE	–	Von Garnier et al. [81]
				IgG2a	+	
				IL-4	–	Astori et al. [88]
				IFN-γ	+	
Wasp venom	BALB/c	Wasp venom +/– Al(OH)$_3$	i.n. Ves v 5 prior sens.	IgG1,IgE	–	Winkler et al. [66]
				IgG2a	–	
				IL-4, IL-5, IFNγ	–	

Examples for mouse models of type I allergy using recombinant allergens. Different sensitization protocols, as well as treatment approaches are listed. Symbols (+, –, =) indicate that the immunological and inflammatory effects in response to allergic sensitization are reduced (–), enhanced (+) or remain unchanged (=) by the respective treatment intervention.

Examples for mouse models of allergic sensitization without treatment procedures (n.d., not done) are also given, for which the effects upon allergen exposure are listed without any scoring.

Abbreviations: i.n., intranasal; i.p., intraperitoneal; s.c., subcutaneous; i.d., intradermal; airwayinflamm., airway inflammation; and AHR, airway hyperresponsiveness. LTB, B subunit of the heat labile enterotoxin of *Escherichia coli*.

with the hypoallergenic Bet v 1 derivative, IL-10 and TGF-β levels were decreased, suggesting operative mechanisms of anergy [69]. Production of hybrid peptides or chimeric molecules, containing the immunodominant sequences of several allergens, may even enhance the efficacy of tolerance induction in polysensitized organisms and may represent a novel form of a "mucosal poly-valent allergy vaccine."

4.3. Induction of immunomodulation—shift from Th-2 to Th-1

DNA-based vaccination represents an attractive alternative to protein-based desensitization. In that respect, it was recently shown that prophylactic vaccination with a recombinant adenovirus expressing a model allergen (β-galactosidase) led to induction of allergen-specific IgG2a antibodies and IFN-γ production in T cells. After subsequent immunization with the antigen, production of specific IgE was abolished, indicating a successful shift from Th-2 to Th1-responses. Suppression of an ongoing IgE response turned out to be the more difficult task and seemed to be strictly dependent on the immunization protocol [99]. Similar results were achieved by vaccination of plasmids encoding the latex allergen Hev b 5 [100], the birch pollen allergen Bet v 1 [101], or the Japanese cedar pollen allergen Cry 1 [102].

Along these lines, application of CpG-oligonucleotides, interacting with the Toll-like receptor 9, also results in strong Th-1 responses [103]. Such CpG motifs have been shown to prevent pulmonary inflammation and Th-2 responses in mice [54,103,104]. In vitro experiments revealed induction of Th-1 responses in PBMCs from allergic patients [105]. Conjugation of CpG-ODNs to allergenic molecules or peptides was more effective than their co-administration [106]. Based on these promising data, the evaluation of CpG-ODNs for treatment of allergic disease in human is in progress.

On the basis of recent epidemiologic and clinical studies, a possible role of certain lactic acid bacteria (LAB) in the prevention of allergic diseases has become evident [107,108]. Recent experimental studies have shown a reduction of IgG1 or IgE when certain LAB were injected or orally applied together with the particular allergen [109–111]. In the mouse model of birch pollen allergy, we could demonstrate that intranasal co-application of certain LAB bacteria with the recombinant Bet v 1, prior and after sensitization with the allergen resulted in a shift from Th-2 to Th-1 responses, characterized by a marked reduction of the IgE/IgG2a ratio accompanied by increased IFN-γ production. We anticipated that local delivery of allergens by recombinant LAB may even enhance the protective/immunomodulatory effects against allergies [112]. Along these lines, a recent study using recombinant LAB expressing the house dust mite allergen Der p 1 could demonstrate the efficacy of such a mucosal allergen-delivery system in reducing allergic immune responses [113].

5. Concluding remarks

It is unquestionable that animal models are important tools for preclinical evaluation of new approaches for prophylaxis and therapy against different diseases including allergies. The results from the experimental studies using alternative routes of antigen administration,

application of recombinant or hypoallergenic molecules, or new antigen-carrier systems to induce counter-regulatory immune responses are generally very promising. However, it is also obvious that in dependence of the experimental model and the antigen used, the effects as well as the mechanisms of action can vary, which might indicate the complexity of predicting clinical consequences of a new therapeutic approach.

The most difficult task therefore remains the step from promising animal studies to successful use in humans, which can be bridged by characterization of immune responses towards potential therapeutic agents in healthy subjects, including studies on the mechanisms in human in vitro systems, prior to application in allergic patients.

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